DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

RECOMBINANT DNA MOLECULE PROGRAM ADVISORY COMMITTEE

MINUTES OF MEETING

JANUARY 15-16, 1977

The Recombinant DNA Molecule Program Advisory Committee was convened for its seventh meeting at 9 a.m. on January 15, 1977 in the Courier-Emissary Room, Tower 1, Sheraton-Four Ambassadors Hotel, Miami, Florida. Dr. DeWitt Stetten, Jr., (Chairman) Deputy Director for Science, and Dr. Leon Jacobs, (Vice Chairman) Associate Director for Collaborative Research, NIH, presided. In accordance with Public Law 92-463 the meeting was open to the public.

Committee members present were:

Drs. Edward A. Adelberg; Roy Curtiss, III; James E. Darnell, Jr.; Peter Day; Donald Helinski; David S. Hogness; Elizabeth M. Kutter; John W. Littlefield; Emmette S. Redford; Wallace P. Rowe; Jane K. Setlow; John Spizizen; Waclaw Szybalski; LeRoy Walters; and William J. Gartland, Jr., Executive Secretary.

A Committee roster is attached. (Attachment I)

The following *ad hoc* consultant to the Committee was present:

Dr. Susan Gottesman, National Cancer Institute, NIH.

The following liaison representatives were present:

Dr. John F. Fulkerson, U.S. Department of Agriculture
Dr. Herman Lewis, National Science Foundation

Other National Institutes of Health staff present were:

Dr. Emmett Barkley, NCI; Dr. Peter Condliff, Fogarty International Center; Dr. Daphne Kamely, NIGMS; Dr. John Nutter, NIAID; Dr. Bernard Talbot, OD; and Dr. Rudolf Wanner, DRS.

Others in attendance for all or part of the meeting were:

Dr. Frederick Blattner, University of Wisconsin; Dr. Harvey Faber, University of Wisconsin; Dr. Robert M. Faust, U.S. Department of Agriculture; Dr. Rae Goodell, Massachusetts Institute of Technology; Dr. James McCullough, Congressional Research Services, Library of Congress; Dr. Lois Miller, University of Idaho; Dr. N.K. Notani, B.A.R.C., India; Dr. Winston Salser, University of California, Los Angeles; Dr. V. Sgaramella, World Health Organization; Dr. Oliver Smithies, University of Wisconsin; Dr. John Tooze, European Molecular Biology Organization, Germany; Dr. Charles Weiner, Massachusetts Institute of Technology; and Dr. W. J. Whelan, University of Miami.

I. Call to Order and Introductory Remarks

Dr. Stetten called the meeting to order at 9 a.m. He summarized the status of the deliberations of the Interagency Committee on Recombinant DNA Research which is to make recommendations on procedures for regulation of the production and use of recombinant DNA molecules both for the Federal and private sectors. This committee already has reached the conclusion that existing regulations are not entirely appropriate.

II. Minutes of September 1976 Meeting

The Committee unanimously voted to accept the Minutes of the September 13-14, 1976, meeting as written with the following amendments:

Page 9, 2nd. paragraph, 3rd sentence and 3rd paragraph, 4th sentence - "There was one abstention" is changed to read "Dr. Curtiss abstained."

Page 10, 2nd complete paragraph, 6th sentence - The word "encouraged" in this sentence is changed to "required.

III. Contracts for Safer Host-Vector Systems

Dr. John Nutter summarized the current status of contract programs of the National Institute of Allergy and Infectious Diseases (NIAID) for the production and testing of safer host-vector systems for the conduct of recombinant DNA experiments. The first contract awards were made in April, 1976. Currently, there are five contracts for the construction of EK2 systems. There have been two meetings of the contractors. As recommended by the Committee, the Research Resources Branch, NIAID will distribute certified EK2 systems.

Dr. Nutter stated that EK2 systems produced under the contract program will be tested for raising to the EK3 level of biological containment. This involves independent confirmation of the genotypic and phenotypic properties of the systems, and environmental testing. Dr. Nutter raised the issue of how EK2 systems constructed independently of the contract program would be tested for EK3 properties. He also pointed out that standards for EK3 testing need to be adopted. Dr. Adelberg pointed out that there are differences in EK2 systems, and that only the best of the EK2 systems should be tested for EK3 properties. The Committee unanimously passed a motion that separate subcommittees should be established for phage and plasmid host-vector systems. These subcommittees would review data and make recommendations to the full committee on EK2 systems, and would advise on which EK2 systems should be tested for EK3 properties without regard to whether the EK2 systems were constructed under the contract program. Not all EK2 systems should be tested for EK3 properties. The Committee recommended that the two subcommittees should keep in close contact with one another. Dr. Stetten stated his assumption that, if an EK2 system fails to meet EK3 criteria, it will revert to EK1 status.

IV. Provision of P4 Physical Containment

There was a lengthy discussion on the provision of P4 physical containment. At the September, 1976 meeting the Committee recommended that a national P4 facility should be established. The National Institute of Allergy and Infectious Diseases was designated as the lead NIH component for establishment and operation of such a facility. This facility would serve a number of functions, including use by visiting investigators, conduct of risk assessment experiments, training in the use of P3 and P4 containment procedures and facilities, and possibly as the site of a mammalian clone bank. Dr. Nutter discussed alternative proposals for P4 containment, such as use of a fleet of mobile laboratories or an array of regional laboratories. He explained that on the basis of six selection criteria a decision was reached to establish a single national facility at the Frederick Cancer Research Center (FCRC). At the September, 1976 meeting, Building 567 at FCRC had been described to the Committee. Since that time more appropriate facilities at FCRC have been indentified (Buildings 467-469). The latter facilities will be able to accommodate 4 to 6 investigators and will permit complete separation of the P3 and P4 laboratories. It will take two years to make these buildings operational. (This also is the revised time-frame for making operational the previously discussed Building 567.) An NIH working group has recommended that for the interim period P4 containment be provided by utilization of Building 550 at FCRC, a mobile high containment laboratory and the renovation of two small P4 modules on the NIH campus in Bethesda.

Dr. Hogness stated that the 10,000 square feet for P4 and 6,000 square feet of P3 laboratory space in the new proposal is probably not required, and that regional facilities make more sense. It was pointed out that multiple facilities require multiple teams of trained staff which greatly increases the cost. In response to a question, Dr. Nutter stated that of 58 investigators who responded to an inquiry, 11 said that they need P4 containment now and 12 said that they will need it in the future. Drs. Darnell and Szybalski stated that P4 containment as described in the Guidelines should be required only for the handling of organisms known to be highly pathogenic, and that P4 for recombinant DNA experiments should be redefined as a P3 laboratory with special containment equipment such as Class III glove-boxes. Dr. Barkley stated that if the hazards of the experiments of high potential risk materialize and approach those of highly pathogenic organisms it would be a mistake to change the definition of P4. Dr. Curtiss stated that, even if the containment levels for certain classes of experiments change in the future, P4 containment always will be required for certain classes of experiments. Dr. Redford pointed out that a decision already has been made that certain classes of experiments require P4 containment and that a P4 facility should be established. He said that the discussion should center on the best way of providing P4 containment. Dr. Kutter stated that there are advantages in a single national facility with a well-trained staff.

Dr. Barkley stated that physical containment standards developed over past decades have been used for the recombinant DNA containment levels, and that it would be a mistake to alter the definitions of physical containment in the absence of biohazard assessment data. A biohazard assessment program can not be carried out without P4 facilities. He said that the Committee ought to recommend both the establishment of a P4 facility and a biohazard assessment program. Dr. Barkley discussed possibilities for providing P4 containment in Building 41 on the NIH campus. Some P4 containment could be made available in 6-9 months, as an interim measure. Dr. Rowe agreed that P4 containment will be needed for the interim period for risk assessment studies. It was also pointed out that there will be other needs for a P4 facility in the future, such as the study of infectious disease agents, perhaps using recombinant DNA technology.

The Committee voted on a motion to reaffirm its recommendation that as expeditiously as possible a national facility should be established with P4 and P3 laboratories to provide space for visiting scientists, and to be utilized for training and risk assessment studies as well as for basic research. Ten members voted in favor of the motion, 4 voted against it.

The Committee unanimously voted on a motion that it is the sense of the Committee that there is a serious need for risk assessment studies and that the Director, NIH should do everything in his powers to provide facilities and personnel to facilitate the onset of these studies.

V. Biological Containment

A. Report of Working Group on Safer Host-Phage Vector Systems

1. Standardized Tests for EK2 Certification

Dr. Szybalski presented a report on the December 13, 1976 meeting of the phage Working Group (Attachment II). He reported that as its first item of business the Working Group had revised and corrected its September 12, 1976, report. The revised report is attached (Attachment III). Dr. Szybalski pointed out the changes and stated that parts I and II of Attachment III should be published as a separate document in NARSM as an operational guide for submission of data on proposed EK2 host-phage systems. The Committee unanimously voted to accept the September 1976 report as modified and corrected.

2. Suitability of DP50 and DP50supF as Hosts for λ EK2 Vectors

Dr. Szybalski discussed the Working Group's report on the suitability of DP50 and DP50supF as hosts for λ EK2 vectors (See II of Attachment II). Dr. Curtiss stated that previously there had been little data to substantiate the properties of DP50. He said that the data now is available, and that Drs. Blattner and Smithies had responded in a satisfactory fashion to all his objections.

3. Review of Data on λgtWES.λB

Dr. Szybalski summarized the Working Group's review of additional data on the λgtWES.λB system of Leder et al. (See III of Attachment II). It is the unanimous opinion of the Working Group that λgtWES.λB together with propagation host stain DP50supF be certified as an EK2 host-vector system. The Committee voted 13 to 0 to accept the Working Group's recommendation. Dr. Curtiss abstained from the vote.

4. Review of Data on the Charon Systems

The Working Group's review of the Charon phage systems of Blattner et al was summarized by Dr. Szybalski (See IV of Attachment II). The Working Group recommended that Charon vectors 3A, 4A, and 316A (also known as 16A), together with propagation hosts DP50 and DP50supF, should be certified as EK2 systems. The Committee voted 12 to 0 to accept this recommendation. Drs. Curtiss and Redford abstained.

The Working Group recommended that Charon vectors 315A, 413A and 414A can not be certified for use in EK2 systems at this time because of the lack of certain data. The Committee unanimously passed a motion to defer consideration of these vectors until additional data is submitted to it through the Working Group.

5. Review of Data on λgt vir Jam 27 Zam 718-λB'

Dr. Gottesman described the phage λgt vir Jam 27 Zam 718-λB' constructed by Sharp and Donoghue, and summarized the Working Group's review of the phage (See V of Attachment II). The Working Group recommended that, subject to receipt of specified additional data, the phage vector should be certified as an EK2 vector without a requirement for using a partially disarmed host for propagation. It also recommended that the phage should be propagated only in closed containers. The additional data were received and distributed prior to the meeting. The Committee unanimously voted to recommend that λgt vir Jam 27 Zam 718- λB' should be certified as an EK2 vector for use in conjuction with E. coli K-12 hosts which do not carry known lambdoid prophages or conjugative plasmids, and with the stipulation that the phage is to be propagated in closed containers.

6. Reconsideration of λgtWES.λC

At its April 1976 meeting, the Committee reviewed data on an EK2 phage system based on the λWES.λC vector of Leder et al. At that time the Committee voted to accept λWES.λC as a vector in EK2 systems with certain general restrictions on hosts which can be used. This was certified as an EK2 system by the NIH on the basis of this recommendation. Concerns subsequently were expressed that the Committee should consider requiring that this and certain other vectors to be used only in conjunction with specific "safer hosts." The Working Group reviewed λWES.λC in light of this recommendation and made the following recommendations regarding the use of λWES.λC (See VI of Attachment II):

> "The overwhelming majority of clones already derived from λWES.λC should be identical to those derived from λWES.λB and should be treated in the same way. Therefore, regardless of any decision about the future use of λWES.λC, we recommend:
>
> 1. DP50supF should be sent to all recipients of λWES.λC, so that clones formed in λWES.λC as with λWES.λB will be propagated in DP50supF.
>
> 2. That individual clones derived from λWES.λC be checked to confirm the absence of the λC fragment.
>
> 3. As soon as λWES.λB and the other vectors recommended in this report are certified, they should also be sent to all recipients of λWES.λC."

These recommendations were discussed by the Committee. It was pointed out that λWES.λC was the first EK2 system to be considered and that it had not been reviewed by a Working Group prior to consideration by the full Committee. Since that time the criteria for EK2 systems based on phage vectors have changed as a result of the deliberations of the phage

Working Group. The Committee voted 14 to 0 to accept the three recommendations of the Working Group with the following added stipulations:

"Recommendation number 2 is modified to read: 'that all clones derived from λWES.λC be checked to confirm the absence of the λC fragment.'

λWES.λC should be decertified as an EK2 system for new cloning as soon as λWES.λB has been certified."

B. Consideration of Safer Host-Plasmid Systems Based on χ1776 and pMB9, pBR313 and pBR322.

Dr. Herbert Boyer of the University of California, San Francisco, and Dr. Stanley Falkow of the University of Washington submitted data on proposed EK2 host-vector systems based on E. coli K-12 strain χ1776 and plasmids pMB9, pBR313 and pBR322. The data were reviewed by an ad hoc subcommittee composed of Drs. Adelberg, Kutter, Setlow, Spizizen and Szybalski. Dr. Adelberg summarized the subcommittee's findings.

Dr. Curtiss presented data from his own laboratory on the transmissibility of pMB9 out of χ1776. Dr. Curtiss' data indicated that the pMB9 cloning vector is transferred out of χ1776 at frequencies 1 to 3 logs lower than the transmission of pSC101 from χ1776 and 4 to 5 logs lower than the transmission of pCR1 from χ1776. The latter already are certified EK2 host-vector systems. Dr. Curtiss concluded that pMB9 in conjunction with χ1776 meets the requirements for an EK2 host-vector system, and provides an additional margin of safety over the already approved EK2 systems because of its lower frequency of transmission. Dr. Kutter presented data on pBR313 and pBR322 which she had received from Dr. Falkow.

The committee recommended by a vote of 13 to 0 that χ1776 (pMB9) should be certified as an EK2 host-vector system. Dr. Curtiss, whose laboratory was involved in the construction of χ1776, was absent from the room during the final discussion and vote.

The Committee voted on a motion that χ1776 (pBR313) and χ1776 (pBR322) should be recommended for EK2 certification on the basis of documentation to be received in writing from Dr. Falkow, compared with the data presented at this meeting and reviewed by the plasmid subcommittee. Ten members voted in favor of the motion; two voted against and Drs. Curtiss and Szybalski abstained. [A Safer Host-Plasmid Working Group which was convened and met in March 1977 recommended that consideration of the systems χ1776 (pBR313) and χ1776 (pBR322) should be postponed pending the receipt of additional data from Drs. Boyer and Falkow concerning mobilization and transmission of these plasmids. The Committee will be asked to review these systems when the additional data become available.]

C. Procedures for Certification

There was discussion of procedures for certification of EK2 systems. One view was that the standing subcommittees should have authority to recommend directly to the NIH the certification of all EK2 systems. Another view was that this procedure should be used only in the case of submissions which involve minor modifications of already approved EK2 systems. A third point of view was that the full Committee must see all the data.

The Committee voted on a motion that the subcommittees be empowered to recommend certification in those cases in which only minor modifications have been made in already certified EK2 systems, that the submissions be sent simultaneously to the full Committee which may raise objections, and that one negative vote of a Committee member would require formal consideration by the full Committee. There were 7 members in favor of the motion, 4 opposed and 3 abstentions.

VI. Biohazard Risk Assessment

A. Workshop

Dr. Rowe discussed the workshop which is being planned to evaluate the postulated hazards of recombinant DNA experimentation. The workshop will deal with areas such as the ecology of *E. coli*, genetic recombination, the biology of plasmids and the pathogenicity of bacteria. Dr. Rowe said that the workshop would have a number of working groups which would consider the following areas:

- experiments to evaluate the biology of *E. coli* carrying non-viral eukaryotic sequences

- the biology of *E. coli* carrying eukaryotic viral sequences

- the biology of inserted sequences (factors affecting translation, etc.)

- the *in vivo* biology of *E. coli* K-12 and its plasmids

- design of experiments to assess the postulated hazards

- consideration of evolutionary arguments (is eukaryotic DNA spontaneously taken up by prokaryotes?, etc.)

Dr. Rowe said that the workshop could make a number of potential contributions, including generation of a source book of data. Dr. Darnell stated that there already is a body of information about *E. coli* K-12, and that a group should be convened to prepare a document to bring together the available data. He proposed that there should be a separate meeting of enteric biologists and gastroenterologists to consider the biology of *E. coli*. The question was raised as to whether representatives of institutional biohazards committees should be invited to the workshop, or whether they should have a separate meeting in parallel.

B. Polyoma Risk Assessment Experiment

The current status of the planning for the polyoma risk assessment experiment of Drs. Rowe and Martin was summarized by Dr. Rowe. He said that, although they will proceed as far as they can with EK2 host-vector systems, a bile salts resistant organism will be needed in order to colonize mice. Therefore, approval to proceed with the experiments at the P4-EK1 level will be needed. The Committee voted on a resolution to recommend that the experiments proposed by Dr. Rowe be permitted, recognizing that they are not in accord with the Guidelines and that the Committee has authority to waive the Guidelines. There were 10 votes for the motion, 1 against and 3 abstentions.

VII. Review of Required Containment Levels

The Committee reviewed a number of requests for clarification of containment levels required by the Guidelines. The Committee recommended that cloning the chorion genes of the silkmoth *Antheraea polyphemus* can be carried out under P2-EK1 conditions provided that the DNA is recovered under aseptic conditions. The basis for this recommendation is that, although the stocks have not been grown in the laboratory for ten generations, the organism is not suspected of being an agricultural pest or disease carrier. It was pointed out that it would require as many as ten years to generate completely inbred stocks at this time.

VIII. Requests for Reduction in Containment on the Basis of Characterization of Clones

The Committee by a vote of 14 to 0 approved a request of Dr. Thomas Maniatis of the Cold Spring Harbor Laboratory to lower containment for a rabbit beta-globin clone on the basis that the clone is rigorously characterized, free of harmful genes and the biological properties of the vector have been maintained.

A number of requests submitted in a petition from Dr. Winston Salser of the University of California, Los Angeles were reviewed. The Committee voted 13 to 0 to approve the requests with certain stipulations. Dr. Redford abstained.

The Committee discussed a request from Dr. Douglas Brutlag of Stanford University School of Medicine to reduce containment levels for bacterial plasmids containing *Drosophila* satellite DNA from P2-EK1 to P1-EK1. Some members of the Committee felt that exceptions to the Guidelines can not be approved, and that the required containment for these experiments is P2-EK1. Other members felt that special consideration should be permissible in reasonable situations. It was pointed out that the satellite DNA consists of a simple well-defined sequence and that the potential risk of these experiments is low. The Committee voted on a motion to deny the request to reduce containment to P1-EK1 on the basis that these levels are below those required by the Guidelines. Seven members voted in favor of the motion, 5 voted against and Drs. Hogness and Redford abstained.

A request from Dr. Robert Crouch of the National Institutes of Health to lower containment levels for a characterized clone of chicken ribosomal DNA was reviewed. The Committee voted 13 to 0 to approve the request on the basis that the clone is rigorously characterized, free of harmful genes and the biological properties of the vector have been maintained. Dr. Redford abstained.

The Committee considered a petition by Dr. Frederick Blattner of the University of Wisconsin to propagate certain characterized mouse globin clones under P3 conditions using phage vector Charon 3A which was recommended for EK2 certification at this meeting. The Committee voted 9 to 0 to approve the request. Three members abstained because they had not had adequate opportunity to study the request.

IX. Definition of Biological Containment

A. Responses to Boston Area Recombinant DNA Group Critique

Dr. Adelberg reviewed the history of a "critique" of E. coli strain χ1776 which had been prepared by the Boston Area Recombinant DNA Group in June 1976. The Director, NIH had requested a point by point response to this document by the ad hoc host-plasmid working group prior to his certification of χ1776(pSC101) and χ1776(pCR1) as EK2 host-vector systems. Dr. Adelberg, who was chairman of the working group, summarized his response and the responses of Drs. Davis, Falkow, Spizizen and Stocker to Dr. Fredrickson. Although they agreed that χ1776(pSC101) and χ1776(pCR1) meet the current criteria for EK2 host-vector systems, there were suggestions to improve the definition of EK2, such as requiring dependence of the plasmid on a specific host. Dr. Szybalski suggested that gradations of EK2 could be defined which would be assigned different levels of physical containment. The latter could be reduced for improved EK2 systems. This would provide incentive for the development of improved EK2 systems. However, some members felt that such a system would make the Guidelines too complicated. The opinion was expressed that the host-plasmid working group might be able to accomplish these goals without revising the Guidelines. There was also sentiment that this proposal is premature, and that further subdivision of containment levels is not desirable at this time. The proposed plasmid working group will take these issues under consideration. Drs. Adelberg, Setlow and Spizizen will represent the full Committee on this working group.

B. Proposed Revision of Biological Containment Criteria

Dr. Adelberg discussed preliminary proposals for revision of the sections of the Guidelines (III-4 to III-16) dealing with biological containment criteria and procedures for testing and certification of EK2 and EK3 systems. The Committee commented on the proposals and referred the document to the host-plasmid working group which will make recommendations to the subcommittee which will be appointed to propose revisions of the Guidelines for consideration at the next meeting. During this discussion the question was raised as to whether proposed EK2 systems should require some animal testing. Some members of the Committee felt that this proposal is against the original spirit of EK2. This matter will be considered further by the host-plasmid working group. The Committee passed a motion by a vote of 8 to 5 to request the National Institute of Allergy and Infectious Diseases to explore mechanisms for providing EK2 rodent testing, when such tests are required.

X. Committee Procedures

The Committee unanimously passed a motion proposed by Dr. Redford that adequate public notice and hearing be provided before adoption of changes in the Guidelines. Dr. Redford also proposed that Committee meetings should be announced more widely.

The Committee voted 12 to 0 on a motion to add a fifth responsibility to the Committee's functions as listed on page IV-6 of the Guidelines, as follows:

> "and (v) recommending to the Director, NIH the approval of exceptions to the Guidelines when such exceptions are, in their opinion, justified by new information or new considerations."

XI. Containment Levels for Experiments Involving Nuclear Polyhedrosis Viruses

Dr. Lois Miller of the University of Idaho made a presentation to the Committee on nuclear polyhedrosis viruses (NPV). She stated that, although the viruses are approved by the Environmental Protection Agency (EPA) for commercial dissemination in the environment as biological insecticides, the NIH Guidelines would require P4-EK2 or P3-EK3 conditions for generation of recombinants in E. coli K-12. She summarized the safety tests required for EPA registration, described the virus structure and cited information which could be expected to be gained from her proposed experiments. She then described the experiments, and proposed levels of containment for the conduct of these experiments. Dr. Rowe expressed concern that much of the information being presented is not published, and that some of the data are out of date. Dr. Day stated that insect viruses are similar to plant viruses, and that perhaps they should not have been included under animal viruses in the Guidelines. Dr. Hogness stated that basically a variance is being requested for experiments with EPA approved viruses.

The Committee separately considered the proposed experiments and requested containment levels. In each case 13 members voted in favor of the motion and one member abstained:

- Recombination of NPV DNA with E. coli host-vectors should be permitted under P2-EK2 containment conditions.

- Recombination of different registered NPVs with the intent of producing hybrid virus should be permitted under P2 conditions.

- The use of NPV DNA as a vector for recombinant DNA in plant insects should be permitted under P2 conditions.

[These recommendations have been construed to constitute a change in the Guidelines and must be approved by the Director, NIH.]

Dr. Rowe said that inclusion of insect host-vector systems will be considered in revision of the Guidelines.

XII. Definition of Recombinant DNA

The Committee briefly considered the need to clarify the meaning of the phrase "different segments of DNA" in the definition of recombinant DNA in the Guidelines. The Committee by a vote of 13 to 0 passed a motion that experiments involving the cutting and religating of purified viral DNA are not subject to the Guidelines. The Committee by a vote of 13 to 0 passed another motion that the cutting and religating of a homogeneous DNA preparation is not subject to the Guidelines.

The definition of "recombinant DNA" will be considered by the subcommittee to be appointed to revise the Guidelines.

XIII. Exchange of Genetic Information and Expression in Prokaryotes

Dr. Helinski presented a report on gene exchange between distally related prokaryotes (Attachment IV). It has been pointed out that there appears to be a discrepancy between the P2-EK1 containment levels required for the cloning of DNA from lower eukaryotic organisms and the P3-EK1 or P2-EK2 levels required for cloning DNA from prokaryotes that do not exchange genetic information with E. coli. Dr. Helinski said that these levels were selected on an assessment of the probability of expression of the genes in E. coli. He said that evidence is accumulating that a large barrier to gene exchange among prokaryotes does not exist. Dr. Hogness said consideration should be given to lowering the containment levels for non-pathogenic prokaryotes that do not exchange genetic information with E. coli to P2-EK1. Dr. Rowe stated that P2-EK1 probably is not high enough containment because prokaryotic genes are likely to be expressed in E. coli. Dr. Curtiss said that he could not agree or disagree with the arguments because more data are needed, and that information should be solicited from experts in microbial ecology, plant pathology, etc. on the extent of gene exchange in nature. The Committee voted 7 to 2 with 2 abstentions to consider at its next meeting reducing the containment levels for cloning DNA from all non-pathogenic prokaryotes (whether or not they exchange genetic information) into E. coli to P2-EK1.

The Committee was told that there is evidence from 4 laboratories that there is exchange of genetic information from E. coli to B. subtilis and vice versa. Therefore, it would appear appropriate to treat B. subtilis as a prokaryote that exchanges genetic information with E. coli. The Committee by a vote of 11 to 1 passed a motion that the cloning of B. subtilis DNA in E. coli should be allowed under P2-EK1 conditions on the basis of preliminary information that B. subtilis exchanges genetic information with E. coli. On accumulation of further data these experiments will fit into the category of "exchangers."

XIV. Consideration of Correspondence

The Committee considered a number of items of correspondence referred to it by the executive secretary. A number of these letters were referred to the subcommittee for revision of the Guidelines.

XV. Subcommittee on Revision of the Guidelines

The Chairman appointed a subcommittee coposed of Drs. Littlefield (chairman), Barkley, Gottesman, Helinski, Rowe, and Walters to propose revisions of the Guidelines for consideration at the next meeting.

XVI. Next Meeting Dates

The Committee selected the dates of May 14-15, 1977 and August 29-30, 1977 for the next two meetings.

XVII. Adjournment

The meeting was adjourned at 5 p.m., January 16, 1977.

Respectfully submitted,

William J. Gartland Jr.

William J. Gartland, Jr., Ph.D.
Executive Secretary

I hereby certify that, to the best of
of my knowledge, the foregoing Minutes
and attachments are accurate and complete.

June 28, 1977
Date

DeWitt Stetten, Jr., M.D., Ph.D.
Chairman, Recombinant DNA Molecule
Program Advisory Committee
National Institutes of Health

RECOMBINANT DNA MOLECULE PROGRAM ADVISORY COMMITTEE

ATTACHMENT I

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SEPTEMBER 1976

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ATTACHMENT II

REPORT TO THE RECOMBINANT ADVISORY COMMITTEE
FROM THE WORKING GROUP ON SAFER HOSTS AND VECTORS:
LAMBDA PHAGE SYSTEMS

The meeting of December 13, 1976, was convened at 9 A.M. in Conference Room No. 114 of Building 1, N.I.H.

Opening remarks were made by Drs. D. Stetten and W. Gartland.

Items on the Agenda were as follows:

I. Consideration of the September 12, 1976 report of the working group on safer hosts and vectors.

II. Discussion of suitability of DP50 as a host in EK2 systems.

III. Consideration of the λgtWES·λB vector-host system of Leder et al.

IV. Consideration of the Charon vector-host systems of Blattner et al.

V. Consideration of the λgtZJvir·λB' system of Donaghue and Sharp.

VI. Reconsideration of the λgtWES·λC system of Leder et al.

VII. Other business.

The meeting was chaired by Dr. W. Szybalski with exception of item IV chaired by Dr. A. Campbell. The other Working Group members were Drs. D. Botstein, S. Gottesman and A. Skalka.

I. Consideration of September 12, 1976 Report

Corrections were made on the report of the last meeting and the corrected version is attached.

II. Discussion of suitability of DP50 and DP50supF as hosts for λ EK2 vectors.

The subject was introduced by Dr. Susan Gottesman and the following comments represent the unanimous opinion of the Working Group:

It was first suggested during the June, 1976 meeting of the Working Group on Safer Host-Phage Systems that genes cloned in lambda vectors could be more effectively contained by using a partially disarmed host for the propagation of these vectors. This concept was supported by the Working Group which met on September 12, 1976. Use of partially disarmed hosts for propagation would decrease the survival of any plasmid- or prophage-carrying host.

A DP50 host (χ1953, isolated by Pereira and Curtiss) has been proposed by Blattner et al. for use with their Charon vectors as an appropriately disarmed host, with supporting relevant data. Drs. Curtiss and Pereira have submitted additional data on the strain and its behavior in rat feeding experi... It is ... after rev... of this information, that DP50 (and ... derivative prepared ... et al.; see October 29, 1976 data) ... necessary to ... disarmed hosts, to be used for prep... the proposed ...

Point-by-point responses to the concerns ... by Drs. Curtiss and Pereira in their Nov. 1st letter follow:

Concern No. 1: No animal feeding experiments have been done on DP50 or its parent strain.

Response: ... et al., Supplement ... to Application for Certification ... EK2 System ... Tables IV and V and data from Curtiss (... 15, 1976). ... tables in... that DP50 and DP50supF are significantly ... for growth in rat and ... hosts.

Concern No. 2: ... dition of dap mutation ... reduce survival of ΔthyA hosts.

Response: It is ... true that ... improvements could be made in DP... to the ... of DP50 itself as a phage ... The use of ... vector ... substitution of a safer ... host ...

Concern ... of the dap genetic region.

Response: ... it would ... further ... understand everything about ... is not essential to the use of ... however, consider carefully the reversion ... the strains to be used ... Curtiss' comments on the necessity of ... strains in stabs ... than slants should certainly be ... all recipients of ... the necessity for ... Experience ... thus far (Supplement IV of ... et al., ...) has not indicated ... special selection for revertants ... in liquid ... Table III of Supplement IV ... rate in ... bacteria. Finally ... 5 of Supplement IV ... the gut are still dap$^-$, strain ... if ... is not a significant factor in ... of DP50 supF.

In addition, ... once ... this strain is being proposed as the ... host for ... cloned segments in phage vectors will ... as phage lysates ... the absence of viable bacteria. The bacterial strain to be used for ... will be stored separately on stabs or even in frozen glycerol and tested before use. Phage vectors which carry cloned segments will never be stored together with viable bacteria for any ... to allow the accumulation of host revertants likely to favor escape of the cloned segment.

Concern No. 4: Use of a recA host for transfection and propagation.

Response: This suggestion should certainly be taken into account in the future construction of some disabled hosts, but we see no reason to require a recA mutation in all such hosts. Tests have been done with the proposed vectors in rec^+ hosts in the presence of limited homology between phage and host, and the frequencies of stable association fell within the limits required for certification of these EK2 systems. Furthermore, some of the safety features in the currently proposed vectors prevent growth in $recA^-$ hosts.

Responses to the concerns raised in the November 9, 1976 letter of Curtiss and Pereira, follows:

Concern No. 1: Superiority of supF derivative of DP50.

Response: The high phage yields obtained by growth in DP50 suggest that suppression of the amber mutations is not severely limiting for growth. The supF derivative could be recommended as a preferred (but not required) host for the EK2 systems.

Concern No. 2: Necessity for a disarmed suppressor-free host.

Response: Such a host could be useful in testing lambda vectors after cloning. However, since a testing host would be used for a single plaque assay of some appropriate dilution of the lysate containing clones, and would not be used for large-scale propagation, a strain such as E. coli C (suggested by Smithies et al.) is adequate.

SUMMARY

We agree with Drs. Curtiss and Pereira's basic point, that one should have data on the particular strain to be used in an EK2 system. Much of the necessary data is contained in Supplement IV of Smithies et al. Many of the comments on the complexity of the dap locus, although interesting, are not necessarily germane. For evaluation of a vector host, we need only to know what survival to expect of the strain under relevant conditions; we need not understand the reasons for the survival. There are a number of suggestions in the Curtiss-Pereira letter for improvements which might be made in the disarmed host. Since clones will be stored in phage lystes, it is a trivial matter to substitute an improved host whenever one becomes available. Therefore, we can confidently recommend the approval of strains which clearly meet our requirements, without in any way barring the introduction of future improvements.

We are cognizant of the time and effort which have been spent in meeting the somewhat fluid requests of the Working Group on phage EK2 systems. It is our feeling that the result of much discussion and many hours of work has been the construction of phage EK2 systems which clearly meet both the letter and the spirit of the NIH Guidelines.

III. Con[illegible] additional data on [illegible] supplied by Leder et al., [illegible] report.

A. (See line 1, test B1 in data submi[illegible] 30, 1976).

The "Yield of [illegible] phage" is 2.3×10^{10} [illegible] DP50supF host), and thus meets the require[illegible] level of "no less than [illegible]

B1. "Probability of persistent association with the host used for propagation...".

The data presented as the result of these tests (less than 10^{-9} persistent associations per output fragment-containing phage, using the model recombinant, exceed the required limits of 10^{-8} (see Test B1 submitted Nov. 30, 1976). The preferred method of construction of a model recombinant should involve *in vitro* insertion of a selectable marker. Although in this case that procedure was not followed, the known common ancestry between the model recombinant and the vector phage (Enquist *et al.*, 1976, Nature [illegible], 596-59[illegible]) suffice to make the result acceptable.

C2(a). "Persistence of the fragment in a [illegible]ermissive λ-sensitive heteroimmune lysogen." (See data in Test C2(a) [illegible] results submitted on Oct. 29, 1976).

The test was designed to expose the "worst possible" case. Under one condition specified (32°C), the result of [illegible] is not significantly different from that required. Under another condition [illegible] the result of [illegible] is over 1000-fold lower than required. Therefore, the Working Group c[illegible]iders these results acceptable.

C2(b). "Probability that marker rescue will negate safety [illegible]tures..." (See Test C2(b) in data submitted on October 29, 1976).

The results of 1.1-4.6×10^{4} for the right-arm disarmament and 9.7×10^{4} for the left-arm disarmament are not significantly different th[illegible]quired.

RECOMMENDATION:

The Working Group recommended at its meeting of Septe[illegible], that λWES·λB together with a suitable host for propa[illegible], be a[illegible] for certification as an EK2 vector system, subject to rec[illegible] and appro[illegible] specified data. As indicated above, these data have been received and approv[illegible]. Therefore, it is the unanimous opinion of the [illegible] of the Working Group that λWES·λB together with the propagation host [illegible] DP50supF be certified as an EK2 vector system.

IV. Consideration of additional data on Charon [illegible] vector-host systems supplied by Blattner et al. as requested in th[illegible] [illegible]mber 12, 1976 report.

(1) Charon 3A and 4A:

C2(b). "Probability that marker rescue will negate safety features..." (See Supplement II by Blattner *et al.* for data for markers to the right of the cloned segment and Supplements II and III for data for markers to the left.)

In the evaluation of rescue of markers to the left, use of the phage ABJλatt80betλimm210P80QSR80 (DM6), which contains perfect left-arm homology with the vector, constitutes a more stringent test than specified, and use of the ABJatt80betλimm210PλQSR80 (λh80imm21 or imm21Signer) phage, which contains imperfect homology in the A-M region (see Fiandt et al, "The Bacteriophage Lambda", 1971, Fig. 1, p. 334), a less stringent test than specified. The phage specified by the Working Group, which Blattner et al. rightly point out does not presently exist, would be expected to give a value intermediate between the two and thus within the limit set by the Working Group.

The values of 4 to 6×10^{-5} for rescue of markers to the right of the "cloned fragment" are lower than the specified 10^{-4} for total rearming. This result alone is, therefore, adequate to satisfy the criterion of safety as stipulated in the test.

(2) Charon 316A

C2(a). "Persistent association in a non-permissive heteroimmune lysogen...". (See Table I of "Additional Data...by Blattner, Williams and Kiefer" received by the Working Group on December 13, 1976).

This value of 5.4×10^{-5} is lower than the 10^{-4} requested.

C2(b). "Marker rescue experiments". (See Table II of "Additional Data..." received by the Working Group on December 13, 1976.)

Results for this test (1.2×10^{-4} for recovery of markers to the right) are not significantly different from the 10^{-4} required for total rearming as stated in the test and, therefore, just pass for certification.

RECOMMENDATION:

Charon 3A, 4A and 316A

At its previous meeting on September 12, 1976, the Working Group recommended that vectors Charon 3A, 4A and 316A together with a suitable host for propagation, be certified subject to receipt and approval of data specified. Data for the host (see II of this report) and for vectors Charon 3A, 4A and 316A have been received and approved and it is the unanimous opinion of the Working Group that these three vector phages, together with the DP50 and DP50supF propagating hosts should be certified as EK2 vector phage systems.

Charon 315A, 413A and 414A

Additional data requested on September 12, 1976 for vector Charon 413A and 414A were not received.

Charon 315A cannot be certified at this time because no tests have been done with an appropriate "model recombinant". Charon 316A does not serve as a "model recombinant" for 315A because it just barely passed the test (see C2(b) above). The fact that there is more homology between the cloned segment and the immunity marker in 315A as compared to 316A suggests that 315A might fail. The Working Group suggests that the uncertainty could be resolved by construction and testing of an appropriate model recombinant or by direct measurement of the frequency of total rearming.

V. Consideration of the application by P.A. Sharp and D.J. Donoghue for classification of λgt[illegible]·λB' as an EK2 vector.

This application was considered by the Working Group completely within the context of the "standardized" laboratory tests outlined in the September 12, 1976 report. The main point of departure for this vector candidate, as compared with those previously considered, is that this vector phage must be propagated by making plate lysates. Therefore, the entire series of tests were adapted to the use of this medium. The results show that this phage meets the required level in all tests performed and with ordinary laboratory strains of E. coli K-12. One test (C2(a)), "survival of fragment-transferring capacity in a non-permissive host" was performed incorrectly in that a heteroimmune lysogen was not used. This probably resulted from ambiguity in the original wording describing our test, which has been clarified at this meeting (see Item I). Results with a permissive heteroimmune lysogen (see Table 2, last line, on p. 10 of the Application) suggest that the vector might pass the required test.

RECOMMENDATION:

It is the unanimous opinion of the Working Group that, subject to receipt of additional data for Item C2(a), this vector phage should be certified as an EK2 vector without requirement for a partially disarmed host for propagation. The additional data may be approved by one of us (A.S.), such approval to be transmitted to Dr. W. Gartland. The Working Group recognizes the fact that propagation of phage in Petri dishes constitutes a possible safety hazard. This problem is easily circumvented by the use of appropriate flasks. We recommend that this should be specified when the vector is distributed.

VI. Reconsideration of the λgtWES·λC system of Leder et al.

λWES·λB, which the Working Group recommends for certification, was derived from λWES·λC by simple in vitro substitution of the inert "λB" fragment for the "λC" fragment. The outer "cloning" arms of the DNA of these phages are identical and "model recombinants" derived from them would be expected to perform identically in our standardized tests. However, the "C" fragment which is present in unfractionated λWES·λC DNA contains phage genes which promote general recombination (red) and prophage formation (att and int), neither of which should be incorporated into recombinant clones grown on ordinary laboratory hosts. It was appreciation of this fact that promoted certain restrictions on the use of λWES·λC as an EK2 vector at the time when it was certified. These restrictions included a stipulation that the outer arms must be "purified" away from the λC fragment before they could be used for in vitro recombination experiments. Thus, use of this vector required what we would consider biochemical and physical procedures to achieve "EK2-level" biological containment. Although this is consistent with the notion introduced in the "Guidelines" that use of DNAs which are enriched (99%) and free of "harmful genes" may be decreased one step in either physical or biological containment, the Working Group is uncertain whether evaluation of systems which involve such combinations of biological with physical or biochemical containment falls within the responsibilities of this Working Group.

It is our understanding that our responsibility is to evaluate the efficacy of genetic features intrinsic to the host-phage systems and independent of other operations. In this strict definition of biological containment, for certification of EK2 vectors, we have not considered the use of other procedures such as the biochemical and physical steps mentioned above or chemical treatment, such as in the use of chloroform, which increases the containment by a factor of 10^8 or higher. The difficulty that we perceive with λWES·λC, as compared with λWES·λB derived clones, is the possibility of formation of a small proportion of *in vitro* recombinants carrying the λC fragment as well as the cloned segment as a result of the possible 1% contamination of the preparation of outer arms. We have no data on the proportion of such recombinants in a typical experiment or their behavior in our EK2 tests (developed subsequent to the certification of λWES·λC). On the other hand, we can see some convenience and potential safety advantage to λWES·λC over λWES·B in that λWES·λC can be grown (before cloning) by induction of lysogens, thereby reducing any possible selective advantage for revertants. These considerations in the absence of data led to disagreement within the Working Group on whether λWES·λC should be recommended for recertification or decertification. However, it is our consensus, in anticipation of the availability of numerous vectors which do pass all the standard EK2 tests, that the use of λWES·λC for the formation of new clones should be discouraged.

The overwhelming majority of clones already derived from λWES·λC should be identical to those derived from λWES·λB and should be treated in the same way. Therefore, regardless of any decision about the future use of λWES·λC, we recommend:

1. DP50*supF* should be sent to all recipients of λWES·λC, so that clones formed in λWES·λC as with λWES·λB will be propagated only in DP50*supF*.

2. That individual clones derived from λWES·λC be checked to confirm the absence of the λC fragment.

3. As soon as λWES·λB and the other vectors recommended in this report are certified, they should also be sent to all recipients of λWES·λC.

VII. Other Business.

(1) The members of this Working Group were asked if they would be willing to continue to serve as 'advisors'. All agreed and another meeting will be scheduled for the beginning of 1977. Items to be discussed in upcoming meetings would include:

(a) Discussion of "Proposal for a policy on modifications generally regarded as safe (GRAS)" by Blattner *et al.*

(b) Ordering of priorities for production and distribution of EK2 vectors for Dr. Nutter.

(c) Consideration of possible tests for phage vectors carrying foreign DNA.

(d) *Consideration of applicability of any in vivo tests for EK2 certification of phage vectors.*

(e) Advice regarding EK3 tests.

(f) How can there be a closer working relationship between the phage and plasmid working groups?

Respectfully submitted,

Waclaw Szybalski, Chairman
Ann Skalka, Secretary

ATTACHMENT III

REPORT TO THE RECOMBINANT ADVISORY COMMITTEE[1]

FROM THE WORKING GROUP ON SAFER HOSTS AND VECTORS:
LAMBDA PHAGE SYSTEMS

The meeting of September 12, 1976, was convened at 12 noon in Conference Room 9, Building 31C of the NIH. Items on the Agenda were as follows:

I. Design of "standardized" laboratory tests for EK2 certification.

II. Modifications in the definition of EK2 phage vectors.

III. Consideration of two applications for EK2 certification of λ phage vectors.

IV. Other business.

I. Standardized Laboratory Tests for EK2 Certification.

Based, in part, on the written suggestions of various members of the Working Group, four criteria were proposed to embody the essential requirements for certification. These criteria were approved unanimously by the Working Group to be recommended for adoption by the Advisory Committee in certification of λ EK2 vector systems.

A. Yield of Vector Phage

The yield of vector phage (+ or - a model cloned fragment) propagated under laboratory conditions should be no less than 10^{10}/ml of unconcentrated crude lysate.

1/ As revised and corrected by the Working Group on December 13, 1976.

B. The probability that a fragment cloned on the vector will form a persistent association with the permissive host used for its propagation and that this complex will then survive for 24 hours outside the laboratory should be less than 10^{-8}. This probability is calculated as the product of (1) and (2) below.

(1) Number of fragment-containing surviving bacteria per output fragment-containing phage in the lysate.

(2) Survival of bacteria after 24 hours under non-permissive test conditions as determined in a separate experiment carried out in the absence of phage.

Comments on:

(1) This fraction should be measured both at the time of lysis and at 24 hours after lysis with the culture maintained under optimal growing conditions. The denominator in both cases is the titer of phage at the time of lysis. The number of fragment-containing bacteria should be measured in an appropriate manner with justification by reconstitution experiments. The test at 24 hours is intended to represent a worst possible case, which should not normally arise. The requirement for a 24-hour test applies only to the host used for propagation, but not necessarily to the host used in procedures at the scale of individual plaques.

As an example, such a test might measure the number of gal^+ bacteria formed after infection of permissive host bacteria (lacking the gal base sequence homology with the cloned fragment) with a phage vector containing a *gal*+ (model cloned DNA) fragment. Demonstration that authentic *gal*+ colonies could be detected at the frequency measured in the test would constitute appropriate justification.

(2) The Working Group does not wish to specify at this time non-permissive conditions that must be met in this test for all cases.

The two tests suggested in current applications: "survival in raw sewage" and "survival in tap water," though useful, refer to survival in nature and are therefore more appropriately considered in the context of EK3 certifications.

For the present, the safety factor characteristic of any particular host can be considered independently for each application. An example of the application of this principle is the host for a current λ EK2 candidate, which in case of culture overgrowth for 24 hours may reach a level of persistent association of about 10^{-6} associations per fragment-containing vector. Thus, production of fragments with these vectors should be limited to hosts offering a factor of safety of at least 10^{-2}. A minimal test for any proposed partially disabled host, however, will include a measure of the reversion frequencies for each of the relevant mutations.

Although killing by chloroform decreases the probability of survival of any clone-containing bacteria by many orders of magnitude, this factor is not included in calculating the degree of biological containment measured in this test.

C. The frequency of survival of fragment-transferring capacity of a vector phage carrying a model fragment should be less than 10^{-8} in the tests specified below.

It is understood that this value (as in B) should represent the product of the following probabilities:

(1) The probability that the phage will survive until it meets a sensitive host.

(2) The probability that (a) the fragment will persist in a non-permissive λ-sensitive heteroimmune lysogen or (b) that the fragment-containing vector phage will acquire genetic material from a related prophage that will serve to negate the safety features of the vector.

Comments:

Data available for wild-type λ suggest that (1) is less than 10^{-3}. Theoretically then, the value for (2) must be 10^{-5} or less. Nevertheless, the Working Group considers it essential that there be some experimental estimation of the "worst possible case." We can very conservatively estimate that the worst possible case (specified below) will actually arise in less than one of ten (10^{-1}) λ-sensitive strains encountered in nature.

This brings the required value for test (2) to 10^{-4}. We therefore recommend that the following "worst-case" tests be included and show:

(a) The number of fragment-containing bacteria per adsorbed input fragment-containing phage should be less than 10^{-4} and

(b) The number of fragment-containing rearmed (wild-type) phage per adsorbed input fragment-containing phage should be less than 10^{-4}. Values of less than 10^{-3} for each of two genetic safety features one located to the left of the cloned fragment and the other to the right, are also acceptable.

It is understood that the host in this worst possible case should be a non-permissive lysogen bearing a heteroimmune prophage which has base-sequence homology with the input phage and a compatible late gene regulatory system.

D. <u>The product of reversion frequencies of "disarming" mutations should be less than 10^{-8}</u>.

<u>Comment:</u>

This test should be done in a non-permissive, non-lysogenic bacteria. Separate determination of the reversion frequencies of individual mutations should be made wherever possible and the product of these should be less than 10^{-8}. Restriction-modification barriers should not be included in this test.

II. Modifications in the definition of EK2 phage vectors.

The Working Group recommends specifically that: Lysogens of any EK2 vector carrying a cloned fragment formed *in vitro* should be considered as EK1 systems.

III. Two applications were considered. Although it was recognized that the data provided were accumulated before the specific tests outlined in I were available, they were discussed and analyzed in the context of these new parameters.

Blattner et al. Application

Re: Vectors Charon 3A and 4A

(a) With respect to the phages themselves, data were presented or provided which could reasonably be considered to meet the tests specified in I, with one exception relating to Item C(2)b, i.e., rearming of the fragment-containing vector by acquisition of genetic material from the prophage of a non-permissive lysogen. Since in this case two types of "disarmament" are employed (with the loss of either rendering the vector "unsafe") each must be independently tested and each must pass the $\pm 10^{-3}$ safety level.

Specifically after infection of the *E. coli* Δ*lac*(*A*λ*B*λh80*att*80*imm*21*QSR*80) host the number of *lac*$^{+}$-containing amber^{+} phage per input *lac*$^{+}$ Charon phage should be less than 10^{-3}. The assays should be performed after the time of lysis normally observed for wild-type λ and without addition of chloroform.

(b) With respect to Item B(2) it is the estimation of the Working Group that the host, DP50, provides a safety factor equivalent to at least 10^{-4} when used for propagation of the vector which carries a cloned fragment. Thus, the requirement stipulated in Item B, even in conditions of overgrowth (test B(1) after 24 hours) where a safety factor of 10^{-6} was obtained, are satisfied when this host is used for propagation.

Re: Vectors Charon 316A, 414A

Data for tests analogous to Item C are not available.

Re: Vectors Charon 315A, 413A

Data for test analogous to Items B(1) and C are not available.

Recommendation

The Working Group recommends: (1) That Charon 3A and 4A be certified subject to receipt and approval of data specified for test of Item C(2)b, as outlined above. This approval may be obtained by ballot of the subcommittee through the mail; (2) That Charon 316A and 414A be certified subject to receipt and approval of data specified in Item C, with approval by ballot through the mail; (3) That Charon 315A and 413A not be certified at this time because of insufficient data.

Leder et al. Application

Re: Vector λWES.λB

Data were presented which were considered to meet the criteria outlined for tests A and D. Indicative data for tests B and C have been presented but complete data for tests B(1) and C are required.

Specifically for B(1): After infection of the permissive host by the model *gal*$^+$ vector phage, the product (1) x (2) should be less than 1[illegible] where:

(1) = number of *gal*$^+$ bacteria per output *gal*$^+$ phage.

(2) = surviving fraction of host after 24 hours.

A DP50*supF* host derived from that used by Blattner et al. may be assigned the same 10^{-4} safety factor allowance in B(2) as DP50 itself. No safety factor for large-scale phage propagation is assigned to the 803-8 strain they describe.

The data submitted for the 803-8 strain justify its use in transfection experiments leading to the formation of single plaque clones.

Specifically for C(2)a:

The host should be a *sup*° *gal*Δ and lysogenic for the heteroimmune lambdoid phage with base sequences derived from λ.

Infection should be made at 30°C and at 37°C.

Specifically for C(2)b:

The host should be a *sup*° lambdoid lysogen.

The number of $\underline{W}^{+}\underline{E}^{+}$ $\underline{gal}^{+}$ phage per input gal-vector should be less than 10^{-3}.

The number of $\underline{gal}^{+}$ $\underline{S}^{+}$ phage per input gal-vector should be less than 10^{-3}.

Recommendation

The Working Group recommends that λWES.λB be approved for certification subject to receipt and approval of data specified above for tests B, C(2)a and C(2)b. This approval may be by mail ballot by the subcommittee.

GENE EXCHANGE BETWEEN DISTALLY RELATED PROKARYOTES

D. Helinski

One of the major classifications of the so-called "eubacteria" is the division of these bacteria into a Gram-positive and a Gram-negative group on the basis of the reaction of a bacterial species to the Gram stain. The separation of bacteria into Gram-positive and Gram-negative groups has a structural basis. The cell wall of gram-positive bacteria consists of a relatively thick layer of covalently-bonded polyssacharide and polypeptide material (the so-called peptidoglycan layer) that provides structural rigidity to the cell. The Gram-negative bacteria as a group, however, have a much thinner peptidoglycan layer and, in addition, possess a relatively thick outer membrane layer that is rich in phospholipid and lipoprotein material.

The transfer of genes between bacteria occurs by transduction (bacterial virus-mediated gene transfer), transformation (uptake of "bare" DNA by a bacterial cell) and conjugation (plasmid-mediated transfer of genes between bacteria that requires cell to cell contact). A reasonable generalization is that virtually all closely related species of bacteria can exchange genes by transduction (inter-species transfer by this process is limited by the relatively narrow host-range of transducing bacteriophage) and transformation (limited between species by [a] the requirement for homology of DNA for most recombination events, excluding transposition events involving insertion elements, [b] the restriction-modification system of a cell when present, and [c] the narrow host-range for maintenance of many plasmid elements). Conjugal mating with the subsequent exchange of DNA can occur between virtually all Gram-negative bacteria (including both naturally-occuring soil and intestinal bacteria) when mediated by a broad-host range plasmid (for example, the P-group plasmids). In the past few years, conjugal mating also has been shown to occur between strains of certain Gram-positive *Streptococcus* species (for example, *Streptococcus faecalis*). To date, however, conjugal mating has not been demonstrated convincingly between any Gram-negative bacterial species and a Gram-positive bacterial species.

In addition to certain barriers that appear to be present and act under certain circumstances to prevent the introduction and establishment of DNA from any bacterial species into any other bacterial species, once DNA is established in a cell it may not be expressed as a result of specificity requirements for the initiation and termination of messenger RNA synthesis and the specificity requirements of ribosome binding, initiation and termination in the case of protein synthesis. Several reports have provided evidence for different specificities for the translation of natural messengers by ribosomes from different species of bacteria. In one report, ribosomes from *Escherichia coli* and *Pseudomonas fluorescens*, two Gram-negative bacteria, translated messenger RNA preparations from all bacteria tested (three Gram-negative species and six Gram-positive species) as well as f2 RNA and T4 early messenger RNA. (M. Stallcup, W. Sharrock, and J. Rabinowitz. Biochem. Biophys. Res. Comm. *58*: 92, 1974). In the same report, ribosomes from *Clostridium pasteurianum*, *Streptococcus faecalis* and *Bacillus subtilis*, three Gram-positive organisms, did not translate messenger RNA preparations derived from any of the Gram-negative organisms, the f2 RNA or the T4 early messenger RNA, but did translate messenger preparations from all six Gram-positive bacterial species tested. The ability of ribosomes from *E. coli* to translate messenger RNA preparations from Gram-positive species of bacteria is consistent with the report of Chang and Cohen (Proc. Nat. Acad. Sci. USA *71*:1030, 1974) who demonstrated the expression in *E. coli* of genetic information carried by DNA of *Staphylococcus aureus*. In this report, genes carried by fragments of *Staphylococcus aureus* DNA, generated by *Eco*RI digestion, were covalently joined to plasmid pSC101 and the resulting plasmid hybrids were established in *E. coli* by transformation. The hybrid plasmid specified a resistance to penicillin that was genetically determined by the *S. aureus* plasmid DNA. It should be noted that in this same experiment these investigators were unable to isolate penicillin-resistant transformants of *E. coli* when the intact *Staphylococcus* plasmid DNA was used to carry out the transformation.

Expression of the *Bacillus subtilis* genes, thymidylate synthetase, (S. Ehrlich, H. Bursztyn-Pettegrew, I. Stroynowski and J. Lederberg. Proc. Nat. Acad. Sci. USA *73*:4145, 1976; C. Duncan, G. Wilson, and F. Young. Gene, in press) and the leucine operon gene isopropylmalate synthetase (K. Sakaguchi, personal communication) in *E. coli* when cloned on *E. coli* plasmids also has been demonstrated. In the case of thymidylate synthetase, this *B. subtilis* gene was cloned on plasmids pSC101 and pMB9. The *B. subtilis* thymidylate synthetase gene cloned in *E. coli* retained its ability to transform thymine-requiring *B. subtilis* strains. The transformations of thymine-requiring *B. subtilis* were carried out with the *E. coli* hybrid plasmids carrying the *B. subtilis* thymidylate synthetase gene. The successful transformants upon analysis appeared to be free of *E. coli* plasmid DNA (Ehrlich *et al.*) In one of these reports (Duncan *et al.*), it was estimated that the *Bacillus subtilis* thymidylate synthetase gene was expressed in the *E. coli* cell at the same level as that found for the *B. subtilis* cell. Similarly, the isopropylmalate synthetase gene of *Bacillus subtilis* cloned on the *E. coli* plasmid RSF2124 in *E. coli* was found to be expressed in *E. coli* (K. Sakaguchi, personal communication).

In what must be considered as an important new development, recent work involving the transformation of *Bacillus subtilis* with antibiotic resistance plasmids purified from the distally related *Staphylococcus aureus* has indicated the maintenance of certain of the *S. aureus* plasmids in *B. subtilis* (S. Ehrlich, personal communication). It should also be noted that a recent report (I. Domaradskii, T. Levadnaia, B. Sitnikov and A. Rassadin. Doklady Akademii *226*: 1443, 1976) describes the successful transformation of the Gram-positive *Bacillus subtilis* with purified R plasmid DNA from *E. coli* (F. Young, personal communication). It is clear that, given the importance of this observation with respect to containment levels for experiments involving recombinant DNA formation between Gram-positive and Gram-negative bacteria that more extensive work should be carried out to critically evaluate the ability of certain plasmid elements to be maintained in both Gram-positive and Gram-negative bacteria. Until this is done the issue of plasmid-mediated exchange of genes between Gram-positive and Gram-negative bacteria will remain an open question.

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